Journal of Research on the Lepidoptera 30(1-2):1-13, 1991

The distribution of radiolabeled pigment precursors in the wing patterns of nymphalid butterflies

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Abstract. The incorporation of radiolabeled tyrosine and tryptophan into the wing patterns of six species of Nymphalidae (*Precis coenia*, *Inachis io*, *Aglais urticae*, *Araschnia levana*, *Heliconius charitonia*, and *Agraulis vanillae*) was studied. Tyrosine is the precursor for the pigment melanin (black to brown), while tryptophan is the precursor for the pigments 3-hydroxykynurenine (yellow) and ommochromes (orange to brown). Tyrosine always and exclusively labeled the dark brown and black scales. The red, light brown, and tan portions of the color pattern never labeled with tyrosine suggesting that the browns and earth tones of the nymphalid wing patterns are not melanins. Tryptophan labeled the yellow portions of the wing pattern in *H. charitonia*, and the red, orange, brown and tan portions of the pattern in the other species we studied, suggesting that the pigments in these regions are all members of the tryptophan-ommochrome pathway in the Nymphalidae. In most cases these two pigment pathways appear to exclude each other in single scales, and only one precursor is incorporated in any given scale. But in a few cases, particularly in *A. levana*, both pathways may be active in some scales.

Introduction

The pigments that make up the color patterns of butterflies belong to relatively few chemical families. The vast majority of wing patterns are made up of melanins and ommochromes. Pterins are common in the Pieridae, while papiliochromes, bile pigments and flavonoids occur sporadically in several families (Needham, 1974; Nijhout, 1985). The most common of the pattern pigments, the melanins and ommochromes, are also among the most difficult of all animal pigments to isolate and identify. The melanins are indefinitely large polymers, complexed with proteins, and almost completely insoluble. Attempts to solubilize melanins generally destroy their molecular structure (Needham, 1974). The ommochromes are fairly large organic molecules. Many of them are also complexed with proteins in situ and thus rendered poorly soluble unless harsh methods are used for their extraction (Linzen, 1974).

Melanins range in color from black, through brown and red, to yellow. The colors of ommochromes largely overlap those of melanins, ranging from brown through red to orange-yellow. As a consequence of the considerable overlap in the color of these pigments, and their general resistance to solubilization and characterization, it has proven extremely difficult to determine whether a specific patch of color on a butterfly wing is melanin or ommochrome. Solubility properties cannot distinguish between melanins and poorly soluble ommochromes and are therefore inadequate for their identification. Chemical characterization by chromatography after vigorous extraction is possible (Umebachi, 1980), but this requires sufficiently large amounts of starting material that it cannot be used to identify a pigment in a specific small area of a wing.

In the present paper we explore a method for detecting melanins and members of the tryptophan-ommochrome pathway in situ, by taking advantage of the fact that they are synthesized from different organic precursor molecules (tyrosine and tryptophan, respectively). When a radiolabeled precursor specific for one or the other pigment families is injected into pupae, that pigment becomes radioactively labeled, and its location in the intact wing can be visualized by autoradiography. While this method does not allow one to distinguish among the various ommochromes or among the various molecular forms of melanin, it can identify a given patch of color as containing either melanin, or ommochrome, or both. Autoradiography thus allows us to study the pattern of specific pigments and to determine whether more than one pigment can occur in a given element of the color pattern.

Materials and Methods

The following species were used for these studies, all members of the family Nymphalidae: *Precis coenia*, *Inachis io*, *Aglais urticae*, *Araschnia levana*, *Heliconius charitonia*, and *Agraulis vanillae*. Two radiolabeled amino acids were used in these studies: *L-[methyl-^{14}C]-Tryptophan* and *L-[^{14}C(U)]-Tyrosine* (New England Nuclear). These compounds were dissolved in an insect saline and their pH was adjusted to between 6.8 and 7.0 with phosphate buffer. Volumes of 2 µl, containing approximately 0.1 µCi of radiolabel were injected into pupae 12 to 24 hours before the onset of pigment synthesis. In a few cases (*A. urticae* and *A. levana*), 1.0 µCi of tryptophan was fed to larvae during their final instar. Adults were allowed to harden their wings for about 12 hours after emergence and wings were then prepared for autoradiography as follows. The radiolabeled amino acids were incorporated into the general proteins of the wing cuticle at a low rate and this resulted in a modest but bothersome amount of non-specific background noise in autoradiograms. We were able to remove nearly all of this background radioactivity by stripping the scales onto adhesive plastic. Wings were pressed tightly between two pieces of book-cover plastic (obtainable from office supply stores) with the sticky sides facing each other. When the two pieces of plastic were peeled apart the scales of dorsal and ventral wing surfaces adhered tightly to the sticky surfaces of the plastic while the wing itself was easily peeled away.

The adhesive surface was then covered with thin plastic food wrap (Handi-Wrap II, DOW Chemical). The food wrap side of this plastic sandwich was the pressed against X-ray film (Kodak Diagnostic Film, X-Omat AR) and stored in a freezer at -70 °C for 1 to 3 weeks. After this exposure period the film was developed in an automatic film processor (Konica QX-60A). Prints were made from these autoradiograms. Hence the bright (white) portions of the prints correspond to areas of radiolabel incorporation.

Results

Figures 1-6 show the tyrosine and tryptophan incorporation patterns in the six species of butterflies. In all cases tyrosine appears to be incorporated primarily into the black and dark brown portions of the pattern while tryptophan is incorporated into the red, brownish orange, and yellow portions of the pattern.

In the dorsal fore wing of *Precis coenia* (Fig. 1A, B), for instance, tyrosine incorporation is strongest in the black eyespot and in the black bands that flank the two red bars in the discal cell. There is a fainter incorporation in the outer ring of the eyespot and in the areas that flank the broad white band on the wing, both of which are dark brown. In the hind wing radiolabel incorporation is primarily in the distal half of the central disk of the two eyespots (which is the only portion of the central disk that is black), in the rings around the eyespots, and in one of the narrow submarginal bands. By contrast, tryptophan incorporation in the dorsal fore wing is strongest in the two red bars within the discal cell, and in the white band. Evidently the red is a tryptophan derivative, possibly an ommatin (Butenandt et al., 1960), and the white regions contain a significant amount of a tryptophan derivative as well. Nijhout (1980) noted that the "white" regions of the fore wing of *P. coenia* is actually not pure white but a light buff color and contains a low concentration of what appears to be the same pigment as that which occurs in the red bars. The tryptophan label is also incorporated in all the orange scales in both fore and hind wing, and also in the yellow-brown scales in between the inner and outer ring of the eyespots on the hind wing. Finally, the light brown ground color in the basal half of the wing labels moderately with tryptophan but not with tyrosine, suggesting that, in contrast to the dark brown regions, these light brown areas contain an ommochrome and not a phaeomelanin, as suggested by Nijhout (1980).

Radiolabel incorporation in the ventral pattern of *P. coenia* (Fig. 1C, D) resembles that seen on the dorsal side: Tyrosine is incorporated primarily in the black portions of the pattern and tryptophan in the red-orange portions. The incorporation pattern in the brown portions of the pattern is a bit more difficult to interpret. There is a slight incorporation of both the tyrosine and tryptophan label throughout the background, although the pattern elements of the nymphalid ground plan (Nijhout, 1985, 1991) label only with tryptophan. The actual pattern on the ventral hind wing

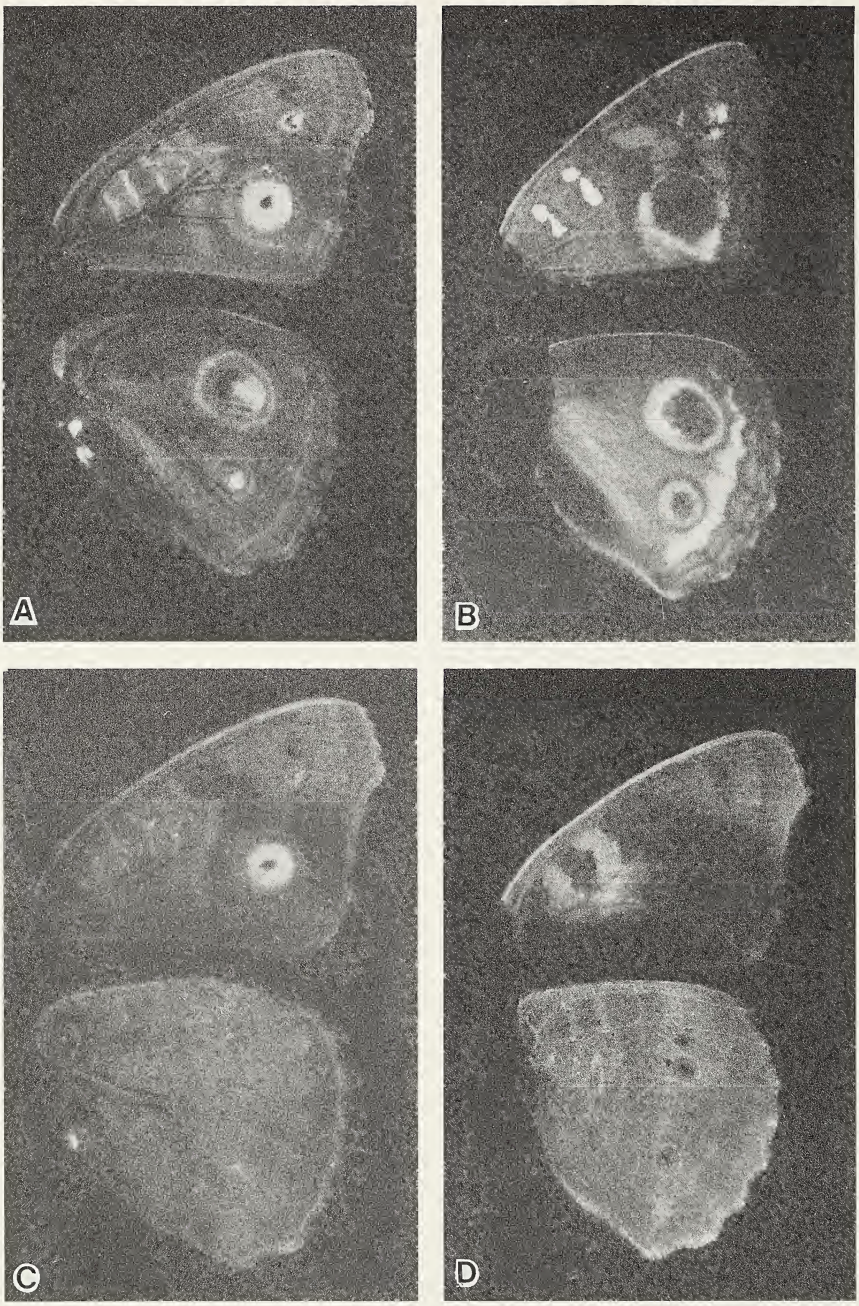

Figure 1. Radiolabel incorporation pattern in the wings of *Precis coenia*. These are negative autoradiograms, so that white areas indicate regions of radiolabel incorporation. A and B, dorsal wing surfaces. C and D, ventral wing surfaces. A and C, tyrosine incorporation pattern. B and D, tryptophan incorporation pattern.

consists primarily of light brown pattern elements superimposed on a tan background. Evidently the pattern elements are a tryptophan derivative (again, probably an ommochrome), while the background contains a mixture of tyrosine and tryptophan derivatives.

The dorsal wing pattern of *Inachis io* (Fig. 2A, B) likewise shows tyrosine labeling in the black portions of the pattern and a very strong tryptophan labeling of the reddish brown portions. The long hairlike scales in the dorsal hind wing label particularly intensively with tryptophan. The white dots on the dorsal fore wing that are the homologs of the foci of the border ocelli (Nijhout, 1985, 1991) do not label with either amino acid, as was the case with the white scales at the center of the fore wing eyespots in *P. coenia* (Fig. 1). The ventral wing pattern consists of a fine black ripple pattern on a dark brown background. Both pattern and background label only with tyrosine, the black more intensely than the dark brown. There is little or no tryptophan labeling of the ventral pattern.

In the wings of *Aglais urticae* (Fig. 3) there are also clear demarcations between areas of tyrosine and tryptophan label incorporation, corresponding to the black and reddish-brown areas, respectively. As in the previous species, the intensity of labeling corresponds to the intensity of pigmentation.

For our studies with *Araschnia levana* we used the summer form, *prorsa* (Fig. 4). The dorsal pattern of this form is mostly black, with a dislocated whitish band running across both fore and hind wing, and with one or two narrow reddish-brown submarginal bands on the hind wing (Fig. 4). The black portions incorporate tyrosine, while the reddish-brown submarginal bands label intensely with tryptophan. In some specimens there is a weak tryptophan labeling of the whitish band (as in the fore wing of *P. coenia*), especially when reddish scales are scattered within the white band. This effect is strongest in forms intermediate between the normal spring and summer forms (Koch, 1991). Again, the small white spots on the dorsal and ventral surfaces that correspond to the foci of the border ocelli are not labeled with either tyrosine or tryptophan.

The ventral color pattern of *A. levana* is more complex with areas of reddish brown, dark brown, black, and white pigmentation. The white portions of the pattern incorporate a small amount of the tryptophan label, as in *P. coenia*, and are not labeled by tyrosine, while the black portions of the pattern are labeled by tyrosine and not by tryptophan. Most of the reddish-brown and dark brown portions of the wing pattern incorporate both the tyrosine and the tryptophan label, although the two labeling patterns do not overlap precisely. Tyrosine labels the reddish-brown areas weakly, and the dark brown areas strongly (and the black areas strongest of all). This is best visible in the ventral fore wing in the region between R_s and $M_{1 \text{ and } 2}$. Thus tyrosine labeling intensity corresponds to the degree "darkness" of the color. Tryptophan labeling, by

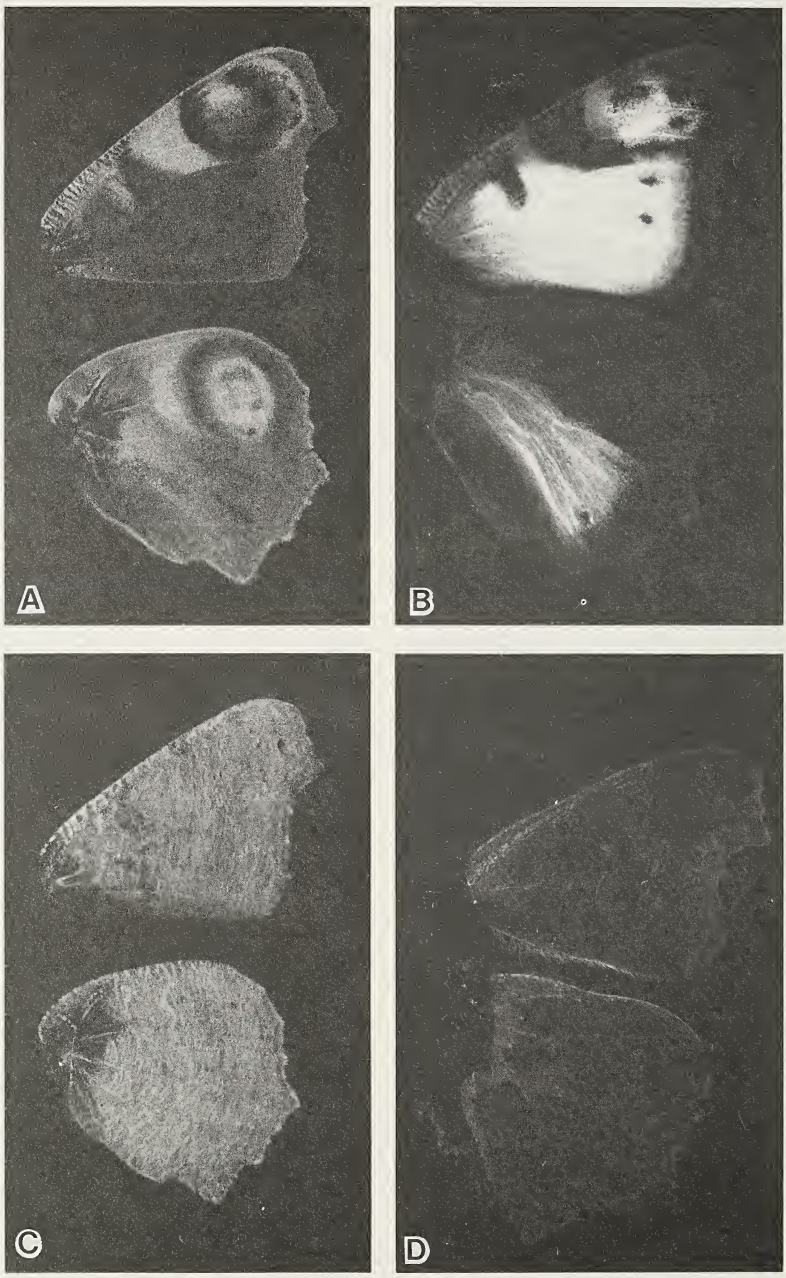

Figure 2. Radiolabel incorporation patterns in the wings of *Inachis io*. Arrangement as in Fig. 1.

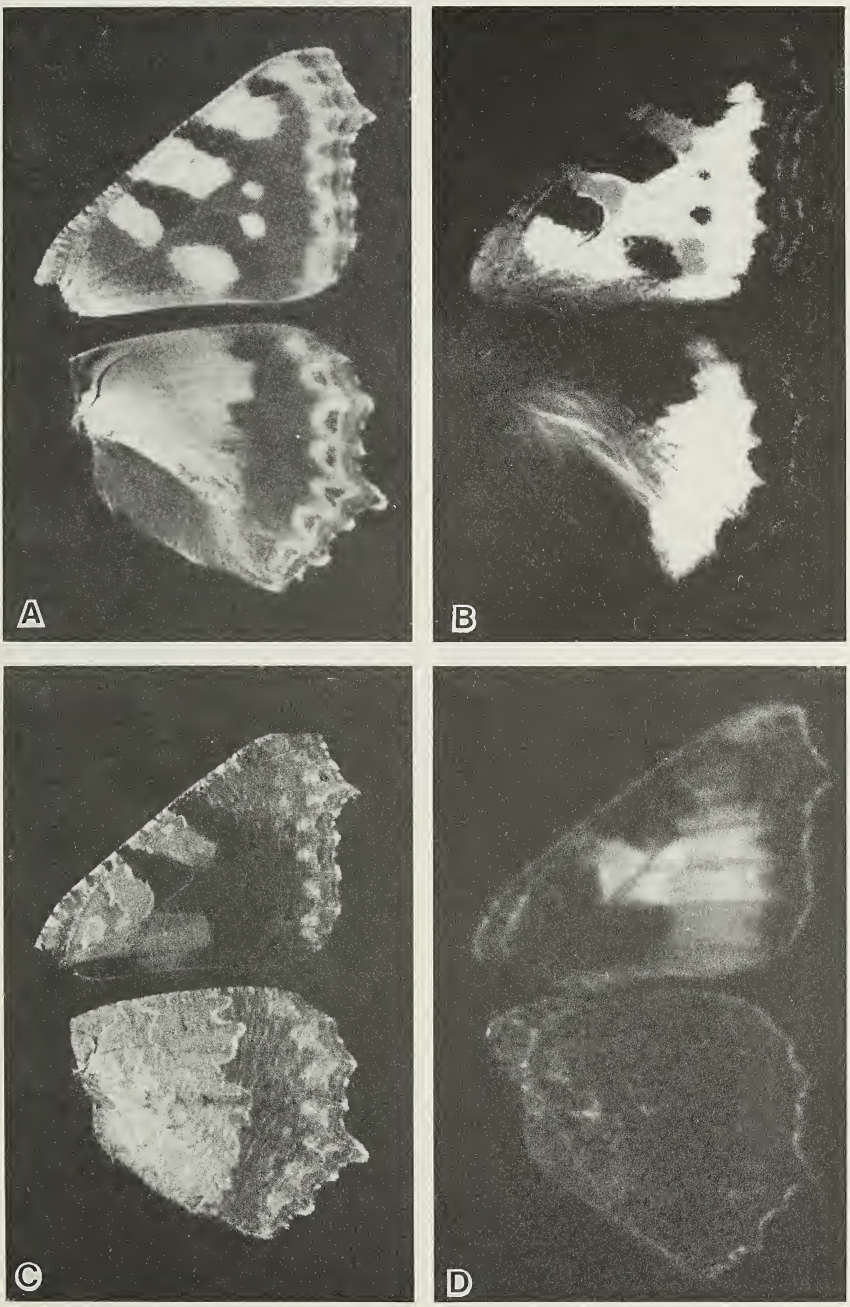

Figure 3. Radiolabel incorporation patterns in the wings of *Aglais urticae*. Arrangement as in Fig. 1.

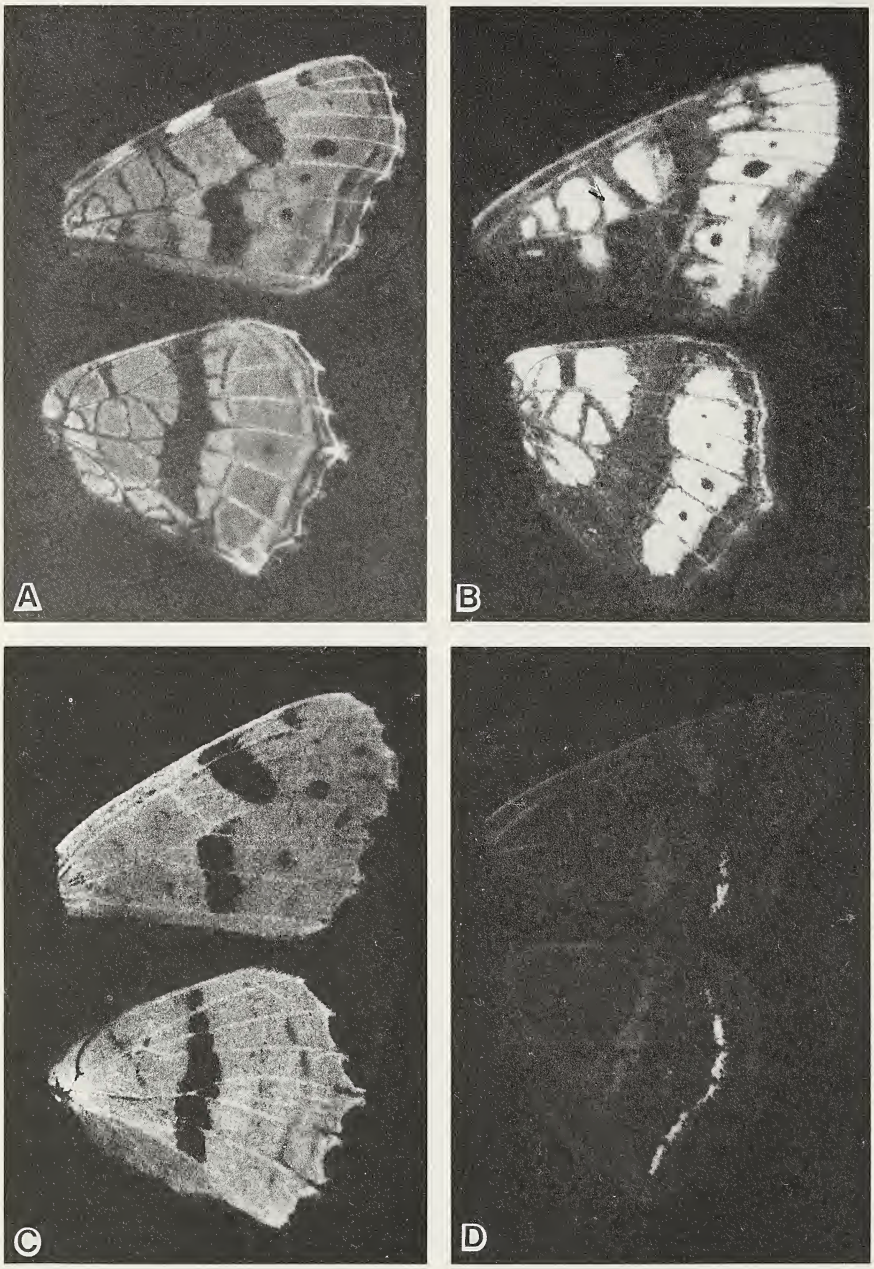

Figure 4. Radiolabel incorporation patterns in the wings of *Araschnia levana*. Arrangement as in Fig. 1.

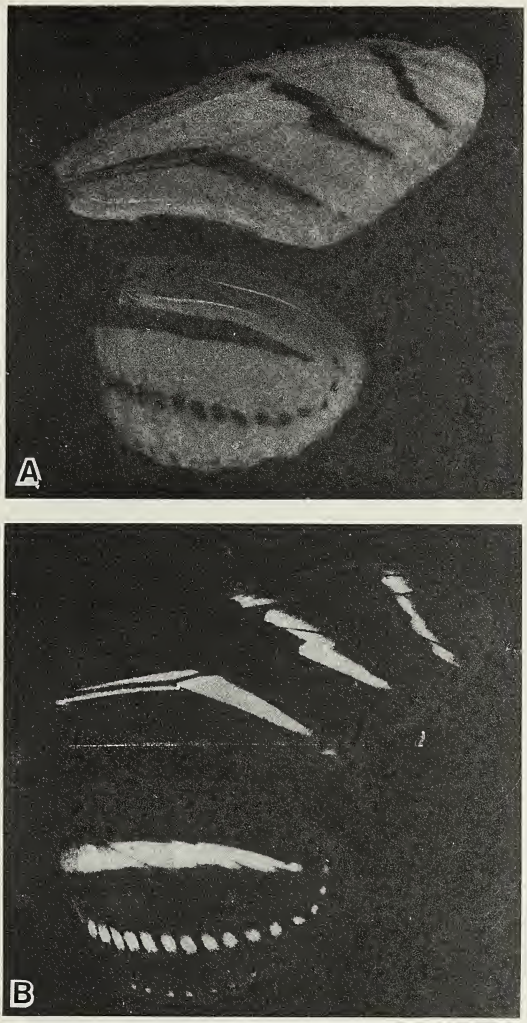

Figure 5. Radiolabel incorporation patterns in the dorsal wing surface of *Heliconius charitonia*. A, tyrosine incorporation pattern. B, tryptophan incorporation pattern.

contrast, is particularly intense in the reddish-brown areas of the pattern, best visible in the proximal third of the dorsal wing pattern and in a broad band corresponding to the field of the border ocelli. This latter field is bordered on both sides by bands that label only with tyrosine (this is particularly easily visible on the hind wing autoradiograms), and correspond to bands of black scales in these areas. By eye, these bands are barely distinguishable from the dark brown field in which they occur. Thus autoradiography is a tool that helps to reveal a differentiation in this portion of the color pattern. It should be interesting to investigate the relation between this biochemical differentiation and the extreme seasonal polyphenism of this species.

The dorsal wing pattern of *Heliconius charitonia* (Fig. 5) is a crisp and bold pattern of black and yellow bands. The black bands are melanin while the yellow bands owe their color to 3-hydroxykynurenine, an intermediate in the ommochrome pathway (Linzen, 1974). Radiolabel incorporation reveals that tyrosine is incorporated exclusively in the black portions of the pattern while tryptophan incorporation is restricted to the yellow bands and the red dots on the ventral side at the base of each wing. There is no overlap of labeling. Thus the black portions of the pattern evidently do not mask a more broadly distributed yellow color. Yellow and black are true alternatives.

Agraulis vanillae shows intense incorporation of tryptophan label in all the reddish-brown areas of the dorsal fore and hind wings, and in the

reddish areas of the ventral fore wing (Fig. 6B, C). Tyrosine labels all the black portions of the pattern. Particularly striking in this species is the intense tyrosine labeling of the black scales that overlie the major veins on the dorsal fore wing (Fig. 6A). The ventral hind wing labels fairly homogeneously but not very heavily with tyrosine. The silver spots on the ventral hind wing do not show any differential label incorporation. The silver spots label as intensely as the light brown "background", which suggests a homogeneous pigmentation of this wing surface behind the structural coloration that is responsible for the silver.

Discussion

It is evident from the foregoing that radiolabeled tyrosine and tryptophan are incorporated differentially into the wing patterns of butterflies. Tyrosine, a precursor for melanin, is associated with the black and dark brown portions of the color pattern. In the Nymphalidae there are no other known pigments for which tyrosine is a precursor, and since in several nymphalid species the tyrosine label is uniquely associated with the black portions of the pattern, we may adopt the working hypothesis that radioactive tyrosine labels only the melanins. Tyrosine is also a precursor for the papiliochromes (Umebachi, 1985), but these pigments are restricted to the Papilionidae. In the species that we studied tyrosine labels only the black and dark brown portions of the pattern (and the reddish-brown portions in *A. levana*), but not the light brown, red, or tan portions, which suggests that the browns and earth tones in butterfly color patterns are not all melanins.

Tryptophan is a precursor for pigments in the tryptophan-ommochrome pathway (Linzen, 1974; Needham, 1974; Umebachi, 1980). Radiolabeled tryptophan becomes incorporated in the red, light brown, tan, and off-white portions of the pattern, suggesting that their pigments are members of that pathway, a fact that has been confirmed by pigment extraction and identification in *A. levana* (Koch, 1991). The reds and browns could be ommatins, rhodommatin or ommatin D, all of which have been identified in the wings of Nymphalidae, or it could be xanthommatin which occurs in butterfly scales as a degradation product of (unknown) labile ommatins (Butenandt et al., 1960). In *A. levana* the reddish-brown portions of the pattern are also known to incorporate radiolabeled sulfur (Lüdicke and Plesse, 1970) and glucose (Koch, 1985), which supports the hypothesis that both ommatin D and rhodommatin may be present in these areas. The yellow of *Heliconius* is 3-hydroxykynurenine, (Tocuyama et al., 1967; Brown, 1981; Koch, 1991), a key metabolite in the tryptophan-ommochrome pathway.

In most species we studied the tyrosine and tryptophan labeled non-overlapping portions of the color pattern. This suggests the operation of discrete biochemical and developmental switches for each of these regions of the wing. To switch a region of the color pattern from yellow to black in *Heliconius*, or from red to black in most other species, for

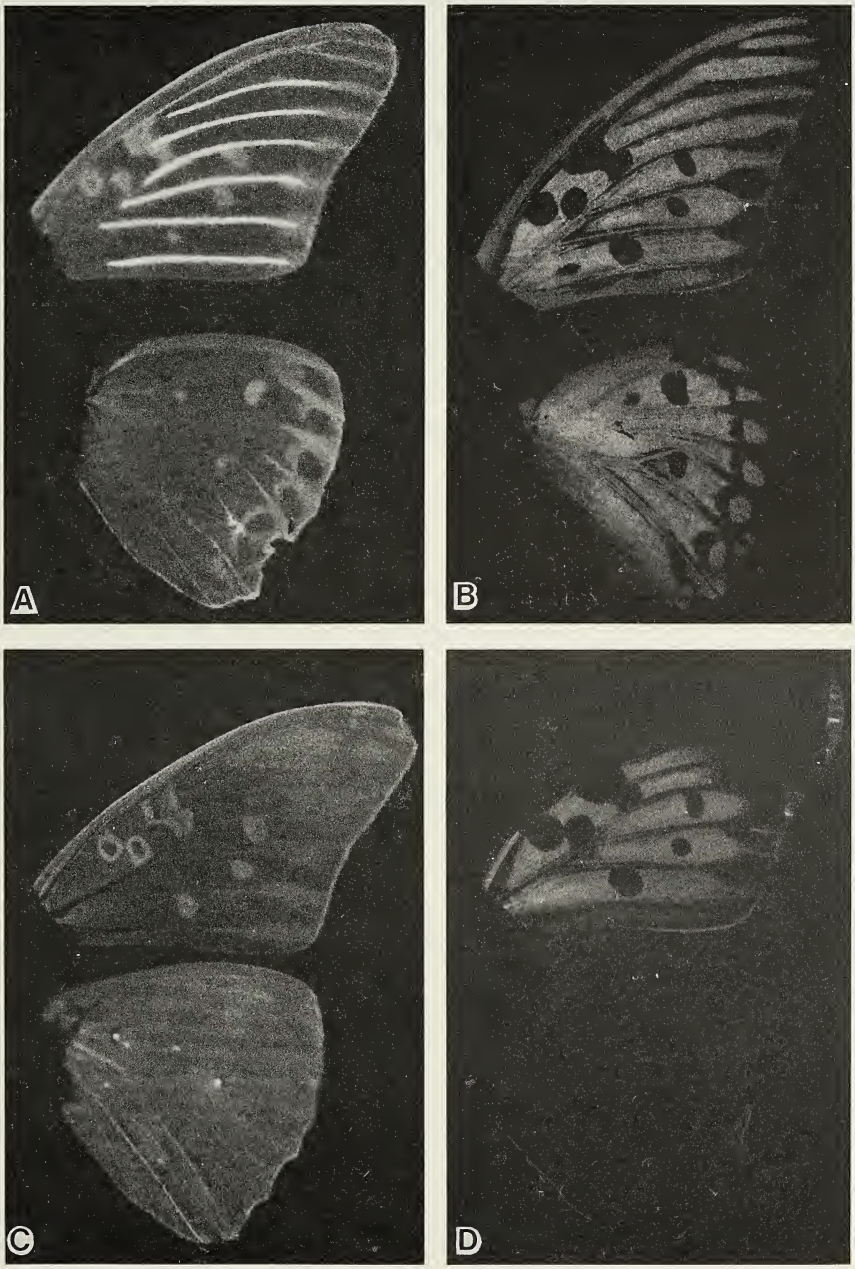

Figure 6. Radiolabel incorporation patterns in the wings of *Agraulis vanillae*. Arrangement as in Fig. 1.

instance, would have to involve the inactivation of critical enzymes in the ommochrome pathway and the synthesis and/or activation of melanin-forming phenoloxidase and its associated proteases. This discrete separation of label is perhaps nowhere better visible than in *H. charitonia* (Fig. 5).

Complex color patterns in butterflies are always composed of a finely tiled mosaic of monochrome scales. Certain color tints, such as the "greens" in Pieridae, as well as graduated changes from one color to another are obtained by locally adjusting the ratios or proportions of scales of different colors (Nijhout, 1985, 1991). In view of this mosaic nature of the color pattern we have long suspected that each scale might only contain a single kind of pigment. Our observations on *H. charitonia* tend to support this notion. We have found several instances in which single yellow scales that developed well within a black region of the wing were labeled with tryptophan, and also instances in which individual black scales within a yellow region were labeled with tyrosine. Thus in this species there appears to be discrete switching of biochemical pathways for pigment synthesis at the level of the individual scale cell. Our autoradiograms do not have sufficient resolution to allow us to determine whether this is true also in any of the other species we studied.

Overlapping tyrosine and tryptophan labeling was found only in *A. levana* and possibly in *P. coenia*. This observation suggests that in these species both melanins and ommochromes can occur in the same scales. We can, however, not at present exclude the possibility that *A. levana* may contain novel pigments that incorporate metabolites of both tryptophan and tyrosine, as is the case in the papiliochromes of the Papilionidae (Umebachi, 1985)

Acknowledgement: This work was supported by a grant from the Duke University Research Council, and, in part, by Grant DCB-8517210 from the National Science Foundation.

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